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14. ABSTRACT

Recent studies have demonstrated the applicability of biomineralization reactions to create an inorganic support matrix suitable to enzyme immobilization. The enzyme/inorganic nanocomposites exhibit excellent mechanical stability and provide an effective method for developing immobilized enzyme reactors, applicable to biocatalysis, biosensors and drug discovery. Enzymes are remarkably versatile catalysts, but in their native soluble form are often labile in the absence of stabilizing agents and are difficult to recover from reaction mixtures. Immobilization of enzymes is therefore frequently employed in an attempt to stabilize enzyme activity and allow reuse of the catalyst. Enzyme immobilization methods primarily involve adsorption, attachment or encapsulation of biomolecules onto or into a solid phase *(1-7).* A range of silicates have been investigated for enzyme immobilization, either by attachment to functionalized mesoporous silica or encapsulation within sol-gel composites, but processing limitations have restricted widespread applicability *(2,4,6,7).*

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Continuous-flow applications of silica-encapsulated enzymes

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Recent studies have demonstrated the applicability of biomineralization reactions to create an inorganic support matrix suitable to enzyme immobilization. The enzyme/inorganic nanocomposites exhibit excellent mechanical stability and provide an effective method for developing immobilized enzyme reactors, applicable to biocatalysis, biosensors and drug discovery.

Enzymes are remarkably versatile catalysts, but in their native soluble form are often labile in the absence of stabilizing agents and are difficult to recover from reaction mixtures. Immobilization of enzymes is therefore frequently employed in an attempt to stabilize enzyme activity and allow reuse of the catalyst. Enzyme immobilization methods primarily involve adsorption, attachment or encapsulation of biomolecules onto or into a solid phase *(1-7).* A range of silicates have been investigated for enzyme immobilization, either by attachment to functionalized mesoporous silica or encapsulation within sol-gel composites, but processing limitations have restricted widespread applicability *(2,4,6,7).*

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Silicification for enzyme immobilization

Silaffin polypeptides in diatoms catalyze the biomineralization of silica to form the exoskeleton *(8,9).* The biosilicification reaction can be mimicked *in vitro* by utilizing synthetic peptides (e.g. R5) based on the native silaffin sequence or from silica-binding peptides identified from combinatorial peptide libraries *(8-13).* Silica formation is also observed in the presence of simple cationic polymers such as polyethyleneimine and by proteins such as lysozyme and silicateins, producing silica nanospheres with a range of morphologies (Figure 1) *(14-18).*

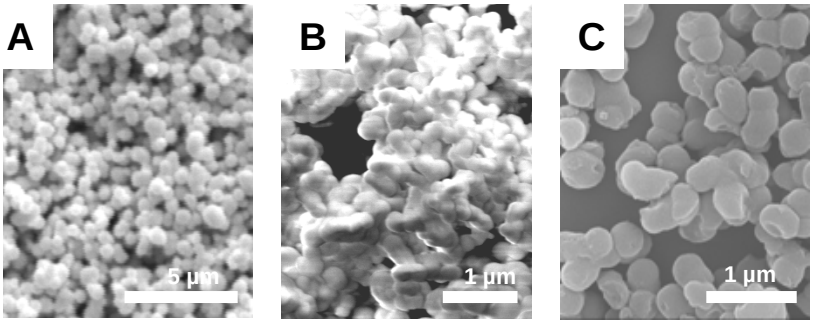

Figure 1. Synthesis of silica nanospheres catalyzed by tetramethylorthosilicate and (a) polyethyleneimine, (b) R5 peptide and (c) lysozyme , as viewed by SEM analysis. (From references 15, 18, 19).

The silica-precipitating species becomes entrapped during the generation of the silica matrix suggesting the potential of the silicification reaction to also encapsulate additional enzymes inside the silica matrix. In practice, the mild encapsulation chemistry and high biocompatibility of the reaction provide a rapid and highly efficient method for immobilizing a wide range of biomolecules (Table I). The biomimetic silicification reaction yields a network of fused silica nanospheres, providing a high surface area for encapsulation and permitting high enzyme loading capacities of up to 20% w/w *(19).*

Table I. Immobilization efficiency of a range of enzymes in silica nanoparticles

Enzyme	*Immobilization efficiency (expressed enzyme activity)*	*Data from Reference*
Butyrylcholinesterase	> 90%	*(19)*
Catalase	100%	*(20)*
Soybean peroxidase	65 - 85%	
Horseradish peroxidase	> 90%	*(20)*
Bromoperoxidase	35 - 48%	
Hydroxylaminobenzene mutase	44 - 67%	*(21)*
Organophosphate hydrolase	25 - 35%	
Nitrobenzene nitroreductase	~ 80% [a]	*(15)*

[a] Immobilized in silica formed from polyethyleneimine and tetramethylorthosilicate (TMOS). All other enzymes are immobilized in silica formed from R5 peptide and TMOS.

The exceptional stability of the silica-immobilized enzymes under operational conditions, dramatically increases the versatility of the biocatalysts. Silica-immobilized butyrylcholinesterase (BuChE), for example, could be stored in aqueous solution at room temperature with no loss of initial enzyme activity, whereas free enzyme under identical conditions lost activity rapidly. The thermostability of the immobilized enzyme was also significantly enhanced. Silica-immobilized BuChE for example retained enzyme activity after heat-treatment of up to 65^{o}C; conditions which caused rapid denaturation of soluble-BuChE. The enhanced enzyme stability can be attributed to the stabilizing effect of the silica support matrix, which prevents the conformational changes typical of enzyme denaturation *(19).*

Biotechnological application to continuous flow systems

A stable immobilized-enzyme preparation is attractive for a wide range of applications, particularly facilitating application to continuous flow-systems. Enzymes catalyze a wide variety of processes that can be exploited for example, in biocatalysis; for the production of novel synthons or drug intermediates. Enzymes also possess a wide range of pharmacological activities and are often investigated for therapeutic effects in drug discovery. Inhibitors of cholinesterase enzymes for example, can be used for the treatment of disorders such as Alzheimer's disease (*22,23*) and nitroreductase enzymes are key activators of prodrugs for cancer therapy *(24,25).* The applicability of silica-encapsulated enzymes was, therefore, further evaluated with respect to the

specific systems described above to provide insight into the versatility of the method.

Immobilized Enzyme Reactors for Cholinesterase Inhibition Studies

Immobilization of enzymes in packed columns specifically designed for continuous flow systems are often referred to as immobilized enzyme reactors (IMERs) *(26-30).* IMERs consisting of immobilized cholinesterase for example have been investigated in drug screening to identify inhibitors for treatment of disorders of the central nervous system, such as Alzheimer's disease. Current IMER configurations however, often exhibit specific drawbacks such as low loading capacity and long preparation times *(31-34).*

An IMER consisting of silica-immobilized BuChE was investigated in an attempt to circumvent some of the current limitations of IMER preparations. Silica-immobilized BuChE was prepared in two alternate column configurations; 1) a fluidized bed and 2) a packed-bed. For the fluidized bed system, substrate conversion was complete for over 12 hours of continuous flow with no loss in enzyme activity or conversion efficiency. The fluidized-bed system could also be operated at higher flow rates with no loss in activity, but with comparably lower conversion efficiency due to a reduced contact time within the column. In the packed-bed system, however, the conversion rate decreased with time. The silica particles became packed under continuous flow conditions, leading to compression and eventual channeling of the silica particles *(19).* Thus, the mechanical stability of the silica-immobilized enzyme was well suited to flow-through systems but the configuration of the column packing required optimization.

In order to avoid the above mechanical limitations, the silica-immobilization technique was modified such that the silica particles form and attach simultaneously to a commercial pre-packed column via affinity binding of a histidine-tag on the silica-precipitating peptide *(35)* (Figure 2). The modified method was used to prepare a butyrylcholinesterase immobilized enzyme reactor (BuChE-IMER). A metal ion affinity chromatography column charged with cobalt ions selectively binds histidine residues on proteins or peptides. A $(His)_6$- homologue of the R5 peptide therefore selectively binds to the cobalt coated surface. The silicification reaction occurs and integrates with the peptide bound to the column, resulting in formation of silica nanospheres attached to the surface of the agarose beads and the concurrent immobilization of the enzyme (Figure 2). The location of the histidine-tag on the silica-nucleating peptide rather than on the protein eliminates any need for recombinant modification of the protein of interest.

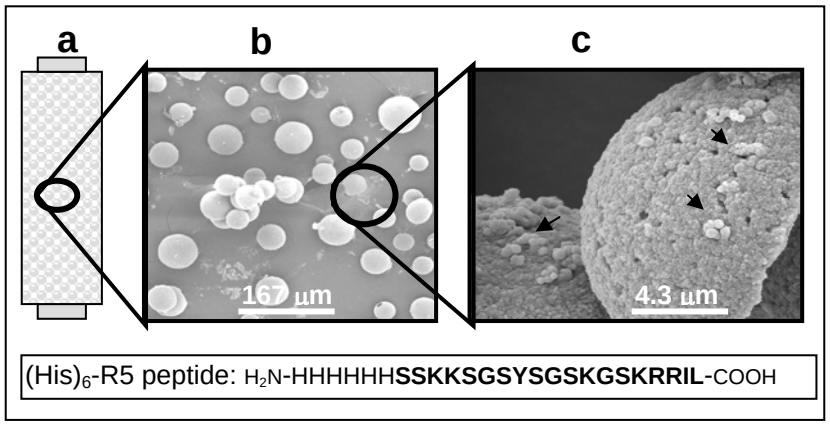

Figure 2. Immobilization of silica beads to agarose beads via affinity binding. A pre-packed affinity chromatography column (a) containing agarose beads (b) is coated with silica (c) by incubation of a histidine tagged-synthetic R5 peptide and TMOS (as viewed by SEM analysis). The $(His)_6$-R5 peptide sequence is shown. Data from reference 35.

The addition of histidine residues at either the carboxyl-terminus or amino-terminus of the silica-precipitating peptide did not affect the silicification reaction. The resulting BuChE-IMER exhibited high loading capacities and an immobilization efficiency approaching 100%. When connected to a liquid chromatography system, the columns could be operated at a wide range of flow rates (up to 3 ml/min) with low back pressure (Figure 3). Multiple substrate injections by means of an auto sampler provided rapid and reproducible analysis, with no significant loss in enzyme activity or conversion efficiency during continuous flow.

The hydrolysis of substrate by cholinesterases is decreased by the presence of inhibitors from which inhibition constant (IC_{50}) values can be derived. The BuChE-IMER can thus be utilized for rapid analysis of inhibition characteristics. A range of cholinesterase inhibitors were investigated and exhibited a concentration-dependent response, from which inhibition constants could be determined (shown in Figure 3b for the inhibitor, Tacrine). The IMER was stable for more than 50 hours of continuous use. In addition, the reusability of the IMER significantly reduces the amount of enzyme required for analysis.

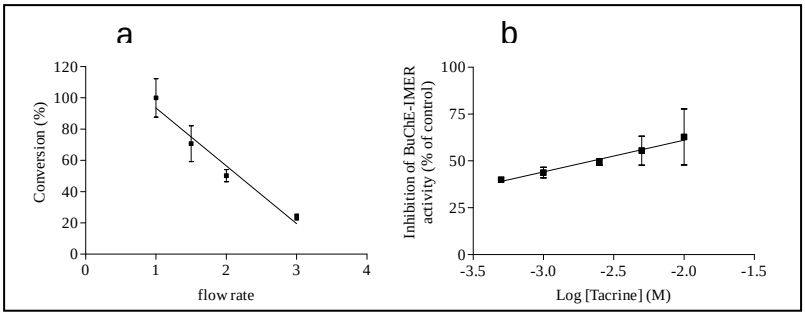

Figure 3 BuChE-IMER activities during continuous flow. Panel (a): Effect of flow rate on the conversion efficiency; (b) Determination of IC_{50} for tacrine using the BuChE-IMER. Data from reference 35

The bioencapsulation strategy described above provides a rapid route for synthesizing IMER systems and provides a model system applicable to a range of formats. The method is also scalable to applications ranging from a microfluidic format for biosensors to large-scale for biocatalysis. Two further examples of silica-immobilized enzymes in packed columns for 1) drug discovery and 2) biocatalysis for drug synthesis will be discussed in more detail below.

Microfluidic Immobilized Enzyme Reactors for Drug Discovery

Nitroreductase enzymes are used to activate prodrugs to a cytotoxic derivative specific to tumor cells (*24,25*). Nitroreductase enzymes catalyze the reduction of a nitro group (strongly electron-withdrawing) to the corresponding hydroxylamine (electron-donating), which results in a large electronic change and provides an effective enzyme-mediated electronic 'trigger'. Nitroreductase enzymes are also known to activate nitrofuran antibiotics by reduction to the corresponding hydroxylamine intermediate, which causes the fragmentation of DNA *(36,37).* Despite the pharmacological relevance of nitroreductases, there are few reports documenting immobilization of such enzymes for drug discovery.

Nitroreductase enzymes were encapsulated in silica formed by a simple cationic polymer; polyethyleneimine (PEI). PEI precipitates silica in a reaction homologous to biogenic systems but with a significant reduction in cost *(15).* The resulting silica particles proved suitable for encapsulation of nitrobenzene

nitroreductase (NbzA) from *P. pseudoalcaligenes* JS45 with immobilization yields of greater than 80% and high retention of enzyme activity (45 – 55 %) *(15).*

The main selection criteria for prodrug formulations are a high affinity for substrate and a differential toxicity between the active species and the prodrug. The affinity of the immobilized-NbzA was therefore determined for nitrobenzene, an anticancer prodrug (CB1954) and a proantibiotic (nitrofurazone). The kinetic properties of the immobilized NbzA were comparable to those of the soluble enzyme, indicating that immobilization was not detrimental to enzyme activity. Nitrofurazone was a poor substrate for NbzA (high K_m value) whereas the K_m value for CB1954 was very low, indicating a high affinity for substrate activation of CB1954 in comparison to other bacterial nitroreductases (Table II).

Table II. Kinetic characteristics of nitroreductase enzymes

	E. coli (NTR)	*P. pseudoalcaligenes JS45 (NbzA)*	
		Soluble	Silica-Immobilized
Nitrobenzene	ND	2.3 (± 0.35)	2.0 (±0.23)
CB1954	862	11.7 (± 1.0)	33.7 (± 4.5)
Nitrofurazone	64	1763 (± 572)	5123 (± 687)

Data from reference 15. All data represent K_m values in μM

As demonstrated previously for encapsulation of BuChE, the silica-immobilization method conferred enhanced stability to NbzA. Immobilized NbzA retained enzyme activity when stored at 4˚C for several weeks and exhibited dramatically higher thermostability than the soluble enzyme *(15).* The enhanced stability in this system is thought to be a consequence not only of the physical support provided by the silica matrix but also the protective nature of PEI itself *(38-40).*

The small size (< 1μm diameter) of the silica-encapsulated NbzA particles provides a high surface to volume ratio considered suitable for microfluidic flow-through systems *(41).* Silica-encapsulated NbzA was therefore packed into a microfluidic device and demonstrated high conversion efficiencies under continuous flow conditions and at a range of flow rates. At 1 μl/min^{-1}, for example nitrobenzene, CB1954 and nitrofurazone were all converted stoichiometrically and conversion of nitrobenzene (>90%) could be maintained for more than 3 days of continuous operation.

Immobilized Enzyme Reactors in Biocatalysis

In whole cells of *Pseudomonas pseudoalcaligenes* JS45, the NbzA described above reduces nitrobenzene to hydroxylaminobenzene (HAB), which undergoes further transformation by HAB mutase to form *ortho*-aminophenol (Figure 4). The activity of NbzA and HAB mutase in concert catalyze the conversion of a range of nitroarenes to yield novel *ortho*-aminophenols *(42,43),* but the use of NbzA for biocatalysis is limited by its requirement for NADPH. The NADPH-dependent reduction of nitroarenes can be replaced however, by a zinc-catalyzed chemical reduction. HAB can then be enzymatically rearranged to *ortho*-aminophenol by HAB mutase, an enzyme with no cofactor requirements.

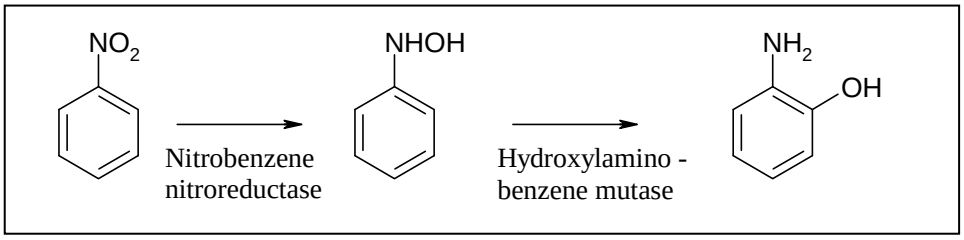

Figure 4. Enzymatic synthesis of ortho-aminophenol

To demonstrate the applicability of the approach, HAB mutase was immobilized and packed into a column and connected in series to a second column containing zinc. Nitrobenzene (1 mM) was pumped through the two columns and *ortho*-aminophenol was produced continuously for over 5 hours with a conversion efficiency approaching 90%. The flow-through system could be operated at higher flow rates and substrate concentration (5 mM at 0.5 ml/min) with a conversion efficiency of approximately 70%, which could be maintained for over 8 hours *(21).*

The use of the zinc/mutase flow-through columns was applied to the formation of a novel antibiotic. The biosynthesis of antibiotics using bacterial cells is limited due to the biocidal properties of the product, for which the immobilized enzyme system provides an attractive alternative. Chloramphenicol contains an active nitro substituent which was converted stoichiometrically to the corresponding aminophenol analog by passage through the zinc and immobilized mutase column in series. At a flow rate of 0.25 ml/min, continuous synthesis of the novel product was maintained for a period of 24 hours *(21).*

Conclusion

Enzyme immobilization methods have been widely investigated for many years, but recent developments in stabilizing enzymes within biomimetic inorganic matrices substantially extends the range of operational stabilities. Nano-sized materials offer a number of intrinsic advantages such as high surface areas which lead to high loading capacities. Silica-encapsulation has proven to be a versatile method for immobilizing biocatalytic activity and is applicable to a wide range of biomolecules. In addition, the morphology of the particles can be controlled by modifying the reaction conditions during silica formation *(13, 44-46).* The primary limitation to broad application is the cost of synthesizing peptides required for silica formation. The use of synthetic polyamines for the silica precipitation reaction however, provides a significant reduction in cost and a realistic opportunity to develop the method for large-scale synthesis of immobilized enzyme preparations. The use of lysozyme to catalyze the silica precipitation *(18)* also imparts the additional benefit of antimicrobial activity to the resulting silica nanoparticles. Lysozyme/silica composites therefore provide antifouling properties to the encapsulated catalysts, for potential use as antibacterial coatings. We anticipate that the resulting silica-encapsulated catalysts will find significant and widespread application in the design of biosensors and for biocatalysis and drug discovery.

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